PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 9/02, 15/52, C07K 14/435, C12N 15/63, 5/06, 5/10, C12Q 1/68 — A2

(11) International Publication Number: WO 00/68364

(43) International Publication Date: 16 November 2000 (16.11.00)

(21) International Application Number: PCT/US00/12527

(22) International Filing Date: 8 May 2000 (08.05.00)

(30) Priority Data:
09/306,538 6 May 1999 (06.05.99) US

(71) Applicants: LUDWIG INSTITUTE FOR CANCER RESEARCH [CH/US]; 605 Third Avenue, New York, NY 10105 (US). THE PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US). MASSACHUSETTS EYE AND EAR INFIRMARY [US/US]; 243 Charles Street, Boston, MA 02114 (US).

(72) Inventors: SIMON, András; Ludwig Institute for Cancer Research, Stockholm Branch, Box 240, S–171 77 Stockholm (SE). ERIKSSON, Ulf; Ludwig Institute for Cancer Research, Stockholm Branch, Box 240, S–171 77 Stockholm (SE). DRYJA, Thaddeus, P.; Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114 (US). BERSON, Eliot, L.; The President and Fellows of Harvard College, 17 Quincy Street, Cambridge, MA 02138 (US). YAMAMOTO, Hioyuji; Massachusetts Eye and Ear Infirmary, 243 Charles Street, Boston, MA 02114 (US).

(74) Agents: HANSON, Norman, D. et al.; Fulbright & Jaworski L.L.P., 666 Fifth Avenue, New York, NY 10103 (US).

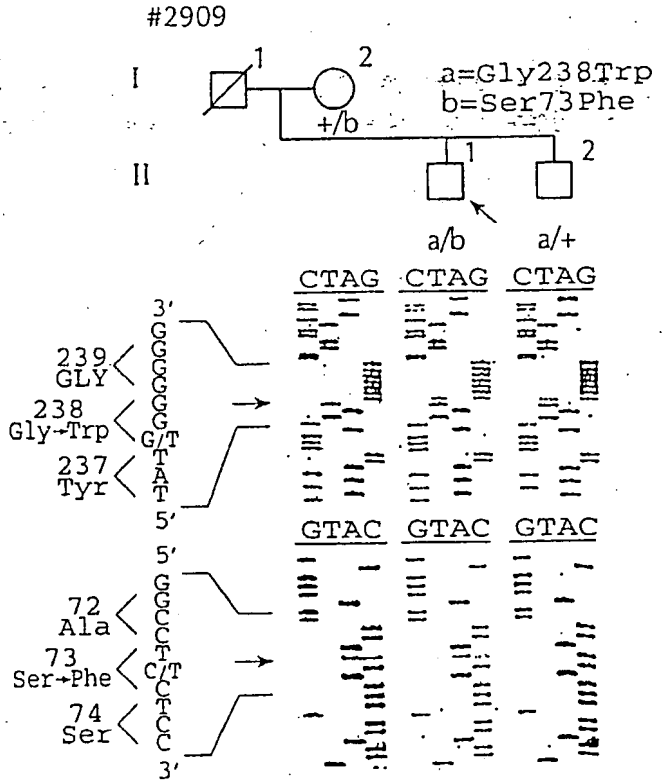
(81) Designated States: AU, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: MUTATIONS IN NUCLEIC ACID MOLECULES ENCODING 11–CIS RETINOL DEHYDROGENASE, THE MUTATED PROTEINS, AND USES THEREOF

(57) Abstract

The invention relates to mutations in the gene encoding 11–cis retinal dehydrogenase. The mutations in the gene and in the resulting encoded protein are correlated to ocular disorders, such as fundus albipunctatus.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

MUTATIONS IN NUCLEIC ACID MOLECULES ENCODING 11-CIS RETINOL DEHYDROGENASE, THE MUTATED PROTEINS, AND USES THEREOF

FIELD OF THE INVENTION

This invention relates to mutations in nucleic acid molecules encoding the protein 11-cis retinol dehydrogenase, or "RDH5," and the resulting mutated protein. These mutations are implicated in ocular disorders, such as fundus albipunctatus. The diagnostic and therapeutic ramifications of these mutations are also discussed and are features of the invention.

BACKGROUND AND PRIOR ART

Retinoids (vitamin A-derivatives) have important physiological functions in a variety of biological processes. During embryonic growth and development, as well as during growth and differentiation of adult organisms, retinoids act as hormones and participate in the regulation of gene expression in a number of cell types. See Lied et al. Trends Genet., 17:427-433 (1992). It is believed that these effects are medicated through two classes of nuclear ligand-controlled transcription factors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), Benbrook et al., Nature, 333:669-672 (1988); Brand et al., Nature, 332:850-853 (1988); Giguere et al., Nature, 330:624-629 (1987); Mangelsdorf et al., Nature, 345:224-229 (1990); Mangelsdorf, et al. Genes Dev. 6:329-344 (1992); Petkovich et al. Nature 330:440-450 (1987); and Zelent et al., Nature 339:714-717 (1989).

Apart from their function as hormones in cellular growth and differentiation, retinoids are also involved in the visual process, as the stereo isomer 11-cis retinaldehyde is the chromophore of the visual pigments. See, e.g. Bridges, The Retinoids, Vol. 2, pp 125-176, Academic Press, Orlando, Florida, (1984).

Under normal physiological conditions most cells, both ocular and non-ocular, obtain all-trans retinol as their major source of retinoids. Despite the many different metabolic events taking place in different tissues, it is known that a common extracellular transport machinery for retinol has evolved. Specifically, in plasma, retinol is transported by plasma retinol binding protein (RBP). See Goodman et al., The Retinoids, Academic Press, Orlando Florida, Volume 2, pp. 41-88 (1984). The active derivatives of retinol, retinoic acid in non-ocular tissues and mostly 11-cis retinaldehyde for ocular tissues, are then generated by cellular conversion using specific mechanisms. To date, none of these mechanisms have been fully defined at the molecular level and several of the enzymes involved have only been identified by enzymatic activities. See Lion et al., Biochem. Biophys. Acta. 384:283-292 (1975); Zimmermann et al., Exp. Eye Res. 21:325-332 (1975); Zimmerman, Exp. Eye Res. 23:159-164 (1976) and Posch et al., Biochemistry 30:6224-6230 (1991).

Polarized retinal pigment epithelial cells (RPE) are unique with regard to retinoid uptake since all-trans retinol enters these cells via two different mechanisms. Retinol accumulated from RBP is taken up through the basolateral plasma membrane, while all-trans retinol, presumably taken up from the interstitial retinol-binding protein (IRBP) following bleaching of the visual pigments, may enter through the apical plasma membrane. See Bok et al., Exp. Eye Res. 22:395-402 (1976); Alder et al., Biochem. Biophys. Res. Commun. 108:1601-1608 (1982); Lai et al., Nature 298:848-849 (1982); and Inu et al., Vision Res. 22:1457-1468 (1982).

The transfer of retinol from RBP to cells is a subject under investigation. In a number of cell types, including RPE, specific membrane receptors for RBP have been identified, which is consistent with a receptor-mediated uptake mechanism for retinol. For example, isolated retinol binding protein receptors, nucleic acid molecule coding for these receptors and antibodies binding to the receptor are known.. These teachings relate to the first of the two mechanisms. See Bavik et al., J. Biol. Chem. 266:14978-14985 (1991); Bavik, et al. J. Biol. Chem. 267:23035-23042 1992; Bavik et

al., J. Biol. Chem. 267:20540-20546 (1993); andU.S. Patent Nos. 5,573,939 and 5,679,772, all of which are incorporated by reference . See also Heller, J. Biol. Chem. 250:3613-3619 (1975); and Bok et al., Exp. Eye Res. 22:395-402 (1976).

Retinol uptake on the apical side of the RPE for the regeneration of 11-cis retinaldehyde ("11-cis retinal" hereafter) is less well characterized. However, regardless of the origin of all - trans retinol, the synthesis and apical secretion of 11-cis retinal seems to be the major pathway for accumulated retinol in the RPE. At present, it is not known whether similar mechanisms are used with regard to cellular retinol uptake through the basolateral and the apical plasma membranes. However, available data show that functional receptors for RBP are exclusively expressed on the basolateral plasma membrane of RPE-cells. Bok et al., Exp. Eye Res. 22:395-402 (1976).

It is also known that retinal pigment epithelial cells (RPE) express a 63 kDa protein (p63). It has also been shown by chemical cross-linking that this protein may be part of an oligomeric protein complex which functions as a membrane receptor for plasma retinol-binding protein (RBP) in RPE-cells, or a component of the retinoid uptake machinery in RPE cells. See Bavik et al., J. Biol. Chem. 266:14978-14875 (1991); Bavik et al., J. Biol, Chem. 267:23035-23042 (1992), and U.S.Patent Nos. 5,573,939 and 5,679,772 The p63 protein has been isolated and the corresponding cDNA cloned. See Bavik et al., J. Biol. Chem. 267:20540-20546 (1993)and the '939 and "772 patents referred to supra. All of these references are incorporated by reference.

11-cis retinal, referred to supra is important in vision, because it is the light sensing chromophore found in cone opsins and rod opsins (i.e., "rhodopsin"), in both cone and rod photoreceptor cells. Deficiencies in vitamin A result in reduction in concentrations of rhodopsin in the retina, which is followed by night blindness. In turn, if night blindness is left untreated it is followed by degeneration of rod photoreceptors, and then cone photoreceptors.In fact, vitamin A supplementation has been reported to slow

the course of retinal degeneration in diseases such as retinitis pigmentosa (Berson et al, Arch. Ophthalmol111:761-772(1993), and to reverse the night blindness found in Sorsby fundus dystrophy, at least temporarily. See Jaconson, et al, Nature Genet. 11:27-32(1995).

Deficiencies in vitamin A can be attributed to one or more causes, including poor diet, or a deficiency in one or more of the proteins involved in transport of vitamin A through the bloodstream. See, e.g., Wetterau, et al., Science 258:999-1001 (1992), and Narcisi, et al., Am. J. Hum. Genet 57:1298-1310 (1995), discussing an inherited deficiency in microsomal triglyceride transfer protein, and Seeliger, et al., Invest. Ophthal Vis. Sci. 40:3-11 (1999), discussing an inherited deficiency in serum retinol binding protein.

Physiological abnormalities and visual symptoms also arise from defects in the storage or metabolism of vitamin A within the retina. With respect to the storage of the vitamin, a number of proteins are thought to bind 11-cis and all-trans vitamin A alcohols and aldehydes in the retina and the retinal pigment epithelium. These proteins include "CRALBP", or cellular relinaldehyde binding protein, "IRBP", or "inter-photoreceptor retinoid binding protein", and "CRBP", or cellular retinol-binding protein. CRALBP and IRBP are known to be essential to photoreceptor physiology, since *null* mutations in the genes encoding these proteins cause photoreceptor degeneration in mammals. See Mau, et al., Nature Genet 17:198-200 (1997); Morimura, et al., Invest. Ophthalmal. Vis. Sci. 40:1000-1004 (1999); Burstedt, et al., Invest. Ophthal. Vis. Sci. 40:995-1000 (1999); Liou, et al., J. Neurasci 18:4511-4520 (1998).

In contrast to the understanding of the pathways and mechanisms discussed supra, abnormalities in the metabolic pathways which convert all-trans retinol from the bloodstream into 11-cis retinal, and that reconvert the all-trans retinal produced after cone and rod photopigments absorb photons of light back to 11-cis retinol are not well understood. Various enzymes are involved in this pathway, and are found in photoreceptor cells, as well as neighboring retinal pigment epithelium, and Müller cells of the retina. Several

of these enzymes have been purified only recently. For example, see Simon et al., J. Biol. Chem 270:1107-1112 (1995), and U.S. Patent No. 5,731,195, both of which are incorporated by reference for teachings relating to purified 11-cis retinal dehydrogenase, and molecules encoding this enzyme, Haeseleer, et al., J. Biol. Chem 273:21790-21799 (1998), for teachings related to all-trans retinal oxido reductase, and Ruiz, et al., J. Biol. Chem 274:3834-3841 (1999), for teachings relating to lecithin: retinal acyltransferase.

It has now been found that ocular disorders are associated with mutations in the nucleic acid molecules encoding 11-cis retinal dehydrogenase (RDH5), and the resulting mutated proteins. These are features of the invention which are set out in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows gel analysis of DNA taken from patients, showing mutation at codon 238 of the RDH5 gene.

Figure 2 shows gel analysis of DNA taken from patients, showing mutations at codons 238 and 73

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

EXAMPLE 1

Given the role of 11-cis retinol dehydrogenase (SEQ ID NO: 1) in vision, it was assumed that patients suffering from hereditary retinal degeneration or malfunction might provide a source of mutated RDH5 genes. Patients with diseases featuring subretinal white or pale yellow spots were chosen for study, because these spots can arise from a lack of vitamin A, and are also found in patients with hereditary retinal degeneration caused by a lack of serum retinol binding protein (Seeliger et al., Invest. Ophthalmol Vis. Sci. 40:3-11 (1999)), or CRALBP (Morimura, et al., Invest. Ophthalmol Vis. Sci. 40:1000-10004 (1999)); Burstedt, et al., Invest. Ophthalmol Vis. Sci. 40:995-1000 (1999)). Twenty nine unrelated patients were evaluated who exhibited retinal

degeneration with subretinal spots (retinitis punctata albescens, or albipunctate dystrophy), two patients were evaluated who suffered from night blindness and subretinal spots (fundus albipunctatus), as were 94 normal controls, 71 patients with recessive retinitis pigmentosa, and 73 with dominant retinitis pigmentosa. The latter two groups were used because there were no assurances that the assumption underlying the study, i.e., that mutations would be found in the RDH5 gene of patients who exhibited subretinal spots, would be correct.

The RDH5 gene contains 4 translated exons. Exon 1 is not translated. Single strand confirmation analysis was used to screen the 4 translated exons, as well as flanking intron sequences.

Genomic DNA was analyzed via polymerase chain reaction. Primers were based upon the sequence of RDH5 published by Simon et al., Genomics 36:424-430 (1996), and Gu, et al., (GenBank Acc. No. AF037062), both of which are incorporated by reference and SEQ ID NO: 2. The primer pairs employed were:

5′- GGCCACAGTA AACTGGACAA-3′
(nucleotides 2301-2320 of SEQ ID NO:2)(sense)

5′- AGCCGGTGAT GAAGACAAAG-3′
(Nucelotides 2458-2477 of SEQ ID NO:2)(antisense)

which amplify exon 2a of the RDH5 gene;

5′- TTACTCTGGG CAGTGCTGTG-3′
(nucleotides 2399-2418 of SEQ ID NO:2)(sense)

5′- AGGACTCGGA AGCCTCTCTG-3′
(nucleotides 2519-2538 of SEQ ID NO:2)(antisense)

which amplify exon 2b;

5'- TTCTGGCACT GCAGCTGGAC-3'
(nucleotides 2499-2518 of SEQ ID NO:2)(sense)

5'- TTCCTGGTGG TCTACCATAC-3'
(nucleotides 2686-2705 of SEQ ID NO:2)(antisense)

which amplify exon 2c;

5'- CCCCAGCATC CTTTTCATCT-3'
(nucleotides 2848-2867 of SEQ ID NO:2)(sense)

5'- GACGCTGGTG ATGTTGATCA-3'
(nucleotides 3038-3057 of SEQ ID NO:2))(antisense)

which amplify exon 3a;

5'- TGAACACAAT GGGTCCCATC-3'
(nucleotides 2969-2988 of SEQ I D NO:2)(sense)

5'- TGTTAGTCCT GGAACCCAGG-3'
(nucleotides 3151-3170 of SEQ ID NO:2)(antisense)

which amplify exon 3b;

5'- AAGAACCCAG CAACTTCGCT-3'
(nucleotides 4030-4049 of SEQ ID NO:2)(sense)

5'- TTCCCTTCAT GTGCCCCTGT-3'
(nucleotides 5247-5266 of SEQ ID NO:2)(antisense)

which amplify exon 4;

5'- CTGATTGCAA CCACCTATGG-3'
(nucleotides 5473-5492 of SEQ ID NO:2)(sense)

5'- AGAGCAGCTT GGCATCCCAA-3'
(nucleotides5619-5639 of SEQ ID NO:2)(antisense)
which amplify exon 5a; and

5'- TAACCAAGGT GAGCCGATGC-3'
(nucleotides 5549-5568 of SEQ ID NO:2))(sense)

5'- CAATCTCTTG CTGGAAGGCT-3'
(nucleotides 5731-5748 of SEQ ID NO:2)(antisense)
which amplify exon 5b.

Amplification of exon fragments was carried out by isolating DNA from leukocytes of subjects, and then adding 20-100 ng of the DNA to 20 μl of a solution containing 20 pM of each pair of the primers described supra, 20 mM Tris-HCl (pH 8.4), from 0.5 to 1.5 mM $MgCl_2$ (explained infra), 50 mM KCl, 0.02 mM of each of dATP, dTTP, and dGTP, and 0.002 mM of dCTP, 0.6 μCi [α-^{32}P] dCTP (3000 Ci/mmol), 0.1 mg/ml bovine serum albumin, 10% DMSO (except for assays on exons 4 and 5a), and 0.25 units of Taq polymerase.

The amount of $MgCl_2$ varied, depending on the primer pair being used, to obtain optimal amplification. In the assays where exons 2a, 2c and 3b were amplified, 0.5 mM was used. When amplifying exons 2b, 3a, and 5a, 1.0 mM was used, and 1.5 mM was used when amplifying exons 4 and 5b. Samples were heated to 94°C for 5 minutes to denature the double stranded DNA, and then from 22-30 cycles of amplification were carried out, with a cycle being defined as 30 seconds of denaturing at 94°C, 30 seconds at 50-60°C for primer annealing, and 30 seconds of extension at 71°C. More specifically, the

annealing temperature for exons 2a,3a,and 5a was 58°C, it was 50 °C for exons 2c and 3b, and 60° C for 2b and 5b The final extension involved heating at 71°C for 5 minutes. In all cases, the pH of the reaction was 8.4.

Amplified DNA was heat denatured, using standard methods, and aliquots of the resulting single stranded fragments were separated through two sets of 6% polyacrylamide gels. One set contained 10% glycerol, and the other did not. Amplification products of the assay for exon 2c were also evaluated by electrophoresis through MDE (mutation detection enhancement) gels. The electrophoresis was carried out for 5-18 hours, at room temperature, and at 8-12 W, before drying and autoradiography. Variant bands (defined as bands migrating at a faster or slower than normal speed through any one of the gels) were evaluated by sequencing the corresponding PCR amplified segments, using standard methods.

The two patients with fundus albipunctatus were found to have missense changes. Specifically, one patient was homozygous for a change in exon 4 at codon 238 (GGG to TGG),(nucleotides 5207-5209 of SEQ ID NO:2) leading to a change in the amino acid encoded by the gene (a change from Gly to Trp), while a second patient was heterozygous for the same change, and for a second change in exon 2 at codon 73 (TCC to TTC)(nucleotides 2589-2591 of SEQ ID NO:2), leading to a change in the amino acid encoded (Ser to Phe). One patient with dominant retinitis pigmentosa exhibited a missense change in exon 2 at codon 33 (ATC to GTC)(nucleotides 2468-2470 of SEQ ID NO:2), leading to an amino acid change (Ile to Val). Silent polymorphisms were also found, at codon 141 (ATC to ATT)(nucleotides 2986-2988 of SEQ ID NO:2) and codon 200 (GTC to GTG)(nucleotides 5093-5095 of SEQ ID NO:2).

The missense changes at 238 and 73 were studied further via familial analysis. For both subjects, the missense change segregated as would be expected if they caused the disease. For example, a sibling of the patient homozygous for the 238 missense change was also homozygous for the change, and was afflicted with the disease, while the patient's mother and two unafflicted siblings were heterozygous. With respect to the patient who was

heterozygous for the missense changes at 238 and 73, there were no affected relatives; however, an unaffected brother was heterozygous for the 238 missense change, and his mother was heterozygous for the 73 missense change. These data are set forth at Figures 1 and 2. It can be seen in Figure 1, for example, afflicted individuals are homozygous for a change at position 238, where "G" has been replaced by "T" in both alleles. In contrast, non afflicted familial individuals are heterozygous for "G" and "T" at this position. The data in Figure 2 show that individuals can be "compound heterozygotes" in that there are single mutations at both positions 238 and 73. What this indicates is that compound heterozygosity can lead to the condition.

The results show that a homozygous mutation at position 238 is indicative of the condition, i.e., fundus albipunctatus. Heterozygous mutations, i.e., situations where only one allele carried the mutation, did not result in the mutation. On the other hand, the data also evidence compound heterozygosity, in that more than one, heterozygous mutation, results in an abnormal condition.

EXAMPLE 2

These experiments describe studies to determine the effect of the missense mutations described supra, i.e., Gly238Trp and Ser73Phe on the activity of 11-cis retinol dehydrogenase.

The first set of experiments was designed to generate mutant forms of the enzyme in vitro.

Human cDNA encoding 11-cis retinol dehydrogenase (RDH5)a is known, as per Simon, et al., supra, and allowed U.S. Patent Application Serial No. 08/258,418, filed June 10, 1994, and PCT WO95/34580 published May 29, 1997, as SEQ ID NO: 14, all of which are incorporated by reference. See SEQ ID NO: 5 as well. The human cDNA molecule described in these references was subcloned into eukaryotic expression vector pSG5, described by Green, et al., Nucl. Acids Res. 16:369 (1988), incorporated by reference. Expression vectors which encoded mutant forms of the enzyme were then generated using single strand mutagenesis, in accordance with Kunkel, et al., Methods

Enzymol. 154:367-382 (1987), and Viera, et al., Methods Enzymol. 154:3-11 (1987), both of which are incorporated by reference. The following primers were used to generate the Ser73Phe and Gly238Trp mutants, respectively:

5'- CTGCAGCGGG TGGCCTTCTC CCGCCTCCAC ACC-3'

(SEQ ID NO: 3)

5'- ACACAGGCCC ACTATTGGGG GGCCTTCCTC ACC-3'

(SEQ ID NO: 4)

In SEQ ID NOS: 3 and 4, codons for the missense mutations are underlined.

Following verification that plasmids carried the introduced mutations, these were used to separately transfect COS-1 cells. As controls, expression vectors expressing wild type 11-cis-retinol dehydrogenase (RDH5), or an empty vector were used. All transfections were carried out using the well known DEAE-dextran methodology, as described by Simon, et al., J. Biol. Chem. 270:1107-1112 (1995), incorporated by reference. An expression vector which expressed β-galactosidase (pSVβ gal), which is commercially available, was cotransfected in each experiment. The transfectants were cultured for 48 hours, under standard conditions, after which they were harvested. Microsomes were prepared from these cells, via gentle homogenization and centrifugation at 7000 x g for 10 minutes, in order to remove debris, and unbroken cells.

Microsomes were collected from supernatant via centrifugation at 100,000 x g for 60 minutes. The microsomes were suspended in PBS, protein concentrations were determined using standard methods, and then aliquots were stored at - 80°C, until used. Equal efficiencies of the transfections were

verified by measuring β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside at 405 nm.

Expression levels of wild type and mutant enzymes were determined via immunoblotting, using pre-existing polyclonal rabbit antiserum, in accordance with the ECL methodology as described by Simon, et al., J. Cell Sci. 112:549-558 (1999).

It was ascertained that both mutants were expressed at levels 8-12 times lower than wild type enzyme.

To monitor the ability of the wild-type and mutant enzymes to catalyze the oxidation of 11-cis retinol to 11-cis retinal, 11-cis retinol, which had been synthesized by reduction of 11-cis retinal with NaBH4 and stored under argon at -80°C, was added to reaction mixtures containing microsomes from the transfected cells. Microsomes containing the wild-type enzyme exhibited the expected ability to catalyze the oxidation of 11-cis retinol to 11-cis retinal in the presence of an excess of the cofactor NAD.

The enzyme reactions, in a total volume of 100µl were carried out in 50mM Tris-HCl buffer pH7.5 containing 5mM NAD or NADH, 50 M 11-cis retinol, or 11-cis retinal, and microsomes at 37 °C. All manipulations of retinoids were performed in dim light. The reactions were stopped by putting the tubes on ice, and the retinoids were immediately extracted with 200µ l n-hexane. Subsequently, 75µ l aliquots were analyzed by reverse-phase HPLC using a C18 column. The mobile phase was acetonitrile/water (85/15, v/v), and the column was eluted under isocratic conditions at a flow rate of 1ml/min. 11-cis retinol and 11-cis retinal eluted at 12.0-12.2 and 14.0-14.3 minutes, respectively. Retinoids were quantified at 320 and 370nm. To measure the Km and Vmax values of wild type 11-cis retinol dehydrogenase, reactions were carried out as above, using a concentration of 11-cis retinol between 0.5 µM and 50 M. In these assays, 2 µg of microsomal protein containing the wild-type enzyme were used in 8-minute incubations. Formed 11-cis retinal was quantified in the HPLC analyses, and the Km and Vmax values were calculated

from Lineweaver-Burk plots. The values were compensated for the extraction efficiency (60-70%, as determined experimentally).

The Km and Vmax values for 11-cis retinol in this reaction were determined to be 6.7 M (average of duplicates 5.5 and 7.8), and 8.4 nmol/mg protein/minute (average of duplicates 7.1 and 9.6)., respectively. These results are consistent with a report by Wang, et al., Biochem. J. 338:23(1999). As expected, the wild-type enzyme was also able to catalyze the reverse reaction, i.e., the reduction of 11-cis retinal to 11-cis retinol, in the presence of an excess of NADH.

The relative activities of the wild type and mutant enzymes were then determined. To do this, microsomes containing a mutant enzyme (20 μg),or wild type enzyme(2µg) together with 18µg of microsomal protein from mock-transfected cells, to standardize the experimental conditions, or no enzyme were combined with 50 mM Tris-HCl buffer (pH 7.5), containing 5 mM of NAD, and 50 μM 11-cis-retinol (forward, oxidation reaction) or 5 mM NADH and 50 μM 11-cis-retinal (reverse, reduction reaction) in a total volume of 100 μl. 50µM 11-cis retinol is approximately a seven-fold higher substrate concentration than the estimated Km value for the wild-type enzyme. The mixtures were incubated for 10 minutes at 37°C, after which the reactions were stopped by putting the mixtures on ice. Retinoids were extracted immediately with 200 μl of n-hexane. Subsequently, 75 μl aliquots were analyzed via reversed phase HPLC using a C_{18} column. The mobile phase was acetonitrile/water (85:15 v/v), and the column was eluted under isocratic conditions at a flow rate of 1 ml/min. The 11-cis-retinol eluted at 12.0-12.2 minutes, and 11-cis-retinal at 14.0-14.3 minutes. Retinoids were quantified at 320 (retinol) and 370 (retinal) nm. Relative activities of wild-type and mutant enzymes were calculated as the ratio of formed 11-cis retinal by transfected versus mock-transfected microsomes. The results were n ormalized for the total extracted retinoid content from each reaction

The wild type enzyme exhibited the expected ability to catalyze the oxidation of 11-cis-retinol to 11-cis-retinal in the presence of an excess of NAD.

Similarly, it catalyzed the reverse reaction, i.e., reduction of 11-cis-retinal to 11-cis-retinol, in the presence of excess NADH.

Both mutants exhibited dramatic reductions in their ability to catalyze the relevant reactions, even though they were used at 10 fold higher concentrations in view of their lower expression levels. The Gly238Trp mutant showed no activity above background level, while the Ser73Phe mutant showed residual activity. Similar results were obtained when catalyzing the reverse reaction

The Gly238Trp mutant was tested further, to determine if it had any activity, by incubating for 20 and 40 minute periods before extracting retinoids. Wild type and Ser73Phe mutant were used as controls. The Gly238Trp mutant showed 2.2 ± 0.14 fold activity above background activity (using mock transformants), as compared to 42.9 ± 4.5 fold (wild type), and 9.1 ± 1 fold (Ser273Phe), after 40 minutes. This indicates that the Ser273Phe mutant has approximately 5 fold less activity, and the Gly238Trp mutant approximately 19 fold less activity than wild type enzyme.

The foregoing examples describe the invention, which relates to mutations in the nucleic acid molecule which encodes 11-cis retinol dehydrogenase, as well as the resulting mutated protein. Specifically, mutations at the codon which encodes amino acid 238 and/or the codon which encodes amino acid 73 are a feature of the invention. In particular, with respect to codon 238 Trp, rather than Gly , is encoded, and with respect to amino acid 73Phe, rather than Ser, is encoded. Also a feature of the invention are molecules where the mutation is at position 33, especially those where Val, rather than Ile is encoded. In addition to the specific mutations described supra, it will be understood by the skilled artisan that, in view of codon degeneracy, more than one type of mutation may result in the specific changes.

As was explained, supra, the mutated forms of the encoding molecules and the proteins are involved in ocular disorders. Hence, a further feature of this invention is the determination of the possible presence of an ocular

disorder, via determining presence of a mutated form of either the nucleic acid molecule, the protein, or both. Such methods include, e.g., hybridization assays such as the polymerase chain reaction, antibody assays involving antibodies specific for an epitope defined by the mutation, and so forth. One specific type of disorder which may be determined via these methodologies is fundus albipunctatus.

Fundus albipunctatus is a form of night blindness wherein patients who suffer from rod photoreceptor malfunction recover after prolonged exposure to darkness. This condition is characterized further by delays in recovery of both rod and cone function after exposure to light; however, fundus albipunctatus is characterized as a form of night blindness, because cone recovery from exposure to light is generally not sufficiently impaired to be subjectively important.

The experiments set forth supra show that the abnormally slow rates of regeneration of code and rod photopigment found in fundus albipunctatus result from slower than normal rates of production of 11-cis retinal, due to reduced 11-cis retinol dehydrogenase activity. Further, decreased, steady state levels of mutant enzymes were seen, suggesting folding or stability problems. Indeed, these two phenomena point to a net capacity of the enzyme to generate 11-cis retinal at one order of magnitude lower than normal. These observations suggest a further application of the invention, which is the treatment of conditions characterized by mutations in the gene encoding 11-cis retinal dehydrogenase, or mutated forms of the protein, by administration of an amount of normal, or "wild type" 11-cis retinol dehydrogenase sufficient to alleviate the disorder. Similarly, since the enzyme is involved in the production of 11-cis retinal, the therapy can also take the form of administration of 11-cis-retinal, in any of the standard forms of ocular administration. Similarly, principles of gene therapy, such as homologous recombination, can be employed to correct the mutation, upon its detection

As the mutated forms of the enzyme are useful in the ways discussed supra, it is desirable to have a ready source of the materials. Hence, another

feature of this invention is the recombinant production of the enzyme, via the use of transformed or transected cells, where the resulting recombinant cells produce the mutant enzyme, as well as multiple copies of the desired nucleic acid molecule.

Mutations in the nucleic acid molecule, in addition to those described above, i.e., at codons for amino acids 238, or 73, or 33, which result in inactive or less active forms of the enzyme, are also a part of this invention. These mutations can include other missense changes, as well as deletions, insertions, frame shift mutations, mutations affecting intron splice donor or acceptor sites, mutations in the promotor region, and so forth. Identification of such mutations via, e.g., PCR, or other forms of assays, are indicative of possible presence of an optical disorder.

Other features of the invention will be clear to the skilled artisan, and are not set forth herein.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

WE CLAIM:

1. An isolated protein comprising the amino acid sequence of wild type retinol dehydrogenase as set forth in SEQ ID NO: 1, with the proviso that (i) amino acid 238 is not Gly or (ii) amino acid 73 is not Ser , or (iii) amino acid 33 is not Ile.

2. The isolated protein of claim 1, wherein amino acid 238 is Trp rather than Gly.

3. The isolated protein of claim 1, wherein amino acid 73 is Phe rather than Ser.

4. The isolated protein of claim 1, wherein amino acid 33 is Val rather than Ile.

5. An isolated nucleic acid molecule which encodes the protein of claim 1.

6. An isolated nucleic acid molecule which encodes the protein of claim 2.

7. An isolated nucleic acid molecule which encodes the protein of claim 3.

8. An isolated nucleic acid molecule which encodes the protein of claim 4.

9. Expression vector comprising the isolated nucleic acid molecule of claim 5, operably linked to a promoter.

10. Recombinant cell comprising the isolated nucleic acid molecule of claim 1.

11. Recombinant cell comprising the expression vector of claim 9.

12. A method for determining possible presence of a disorder of the eye, comprising assaying a sample taken from a subject believed to have an eye disorder for a mutation in the nucleic acid molecule which encodes retinol dehydrogenase, presence of said mutation being indicative of possible presence of said disorder.

13. The method of claim 12, said method comprising polymerase chain reaction.

14. The method of claim 12, wherein said mutation is a mutation at the codon which encodes amino acid 73 of retinol dehydrogenase.

15. The method of claim 12, wherein said mutation is a mutation at the codon which encodes amino acid 238 of retinol dehydrogenase.

16. The method of claim 15, wherein said disorder is fundus albipunctatus.

17. The method of claim 16, wherein said mutation occurs in both alleles of said subject's gene which encodes retinol dehydrogenase.

18. The method of claim 17, wherein both of said alleles carry the same mutation.

19. The method of claim 17, wherein each of said alleles carries a different mutation

20. The method of claim 18, wherein said mutation results in a change from glycine to tryptophan at the codon for amino acid 238.

21. The method of claim 19, wherein one of said alleles carries a mutation resulting in a change from glycine to tryptophan at the codon for amino acid 238, and the other allele carries a mutation resulting in a change at the codon for amino acid 73, resulting in a change from serine to phenylalanine.

FIG. 1

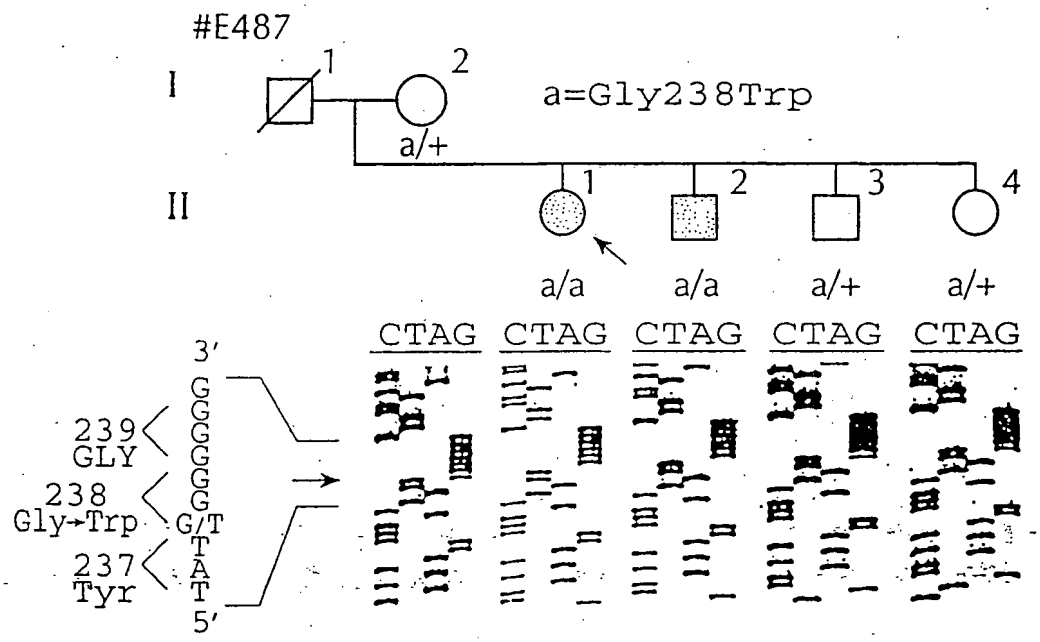
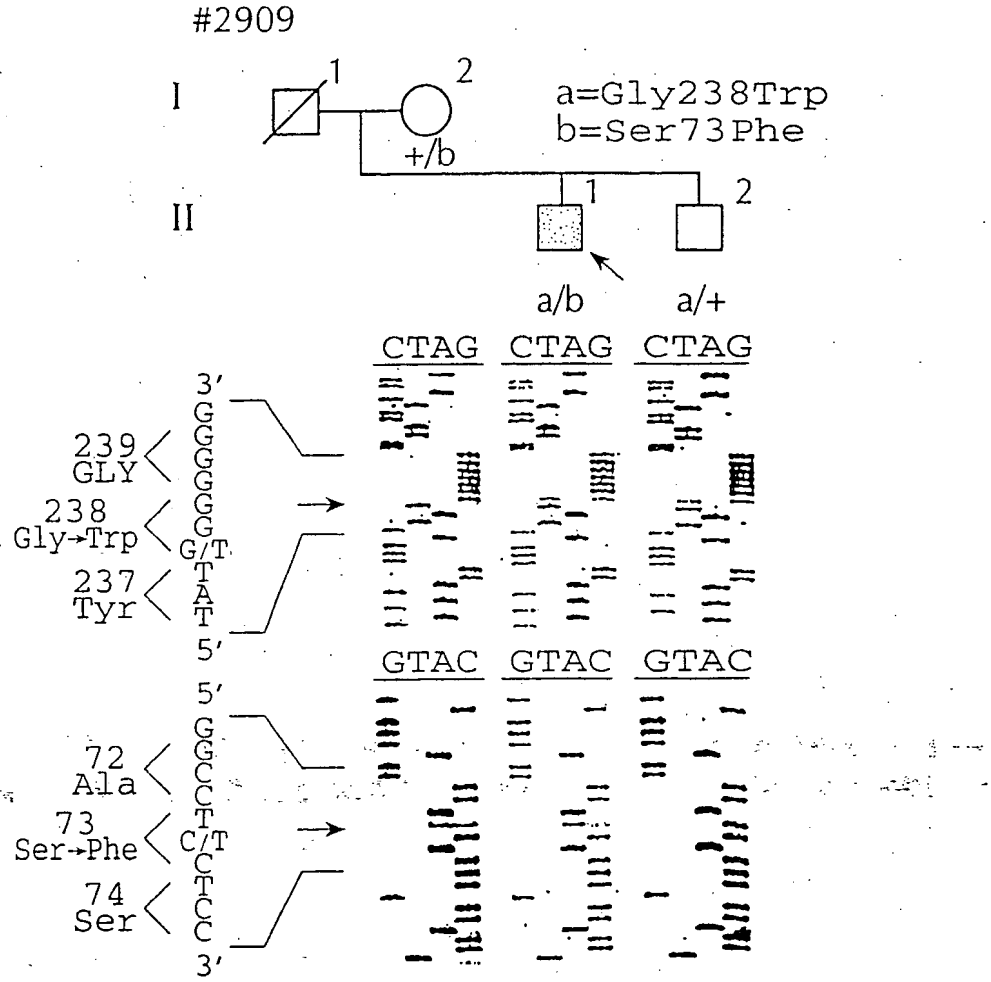

#E487
I
1
2
a/+
a=Gly238Trp
II
1
2
3
4
a/a
a/a
a/+
a/+
CTAG
CTAG
CTAG
CTAG
CTAG
3'
239
GLY
238
Gly→Trp
237
Tyr
G/T
5'

FIG. 2


#2909
I
II
1
2
+/b
1
2
a=Gly238Trp
b=Ser73Phe
a/b
a/+
CTAG
CTAG
CTAG
GTAC
GTAC
GTAC
3'
5'
239
GLY
238
Gly→Trp
G/T
237
Tyr
5'
3'
72
Ala
73
Ser→Phe
C/T
74
Ser

SEQ ID NO: 1

```
Met Trp Leu Pro Leu Leu Leu Gly Ala Leu Leu Trp Ala Val Leu Trp
                5                  10                  15

Leu Leu Arg Asp Arg Gln Ser Leu Pro Ala Ser Asn Ala Phe Val Phe
            20                  25                  30

Ile Thr Gly Cys Asp Ser Gly Phe Gly Arg Leu Leu Ala Leu Gln Leu
        35                  40                  45

Asp Gln Arg Gly Phe Arg Val Leu Ala Ser Cys Leu Thr Pro Ser Gly
    50                  55                  60

Ala Glu Asp Leu Gln Arg Val Ala Ser Ser Arg Leu His Thr Thr Leu
65                  70                  75                  80

Leu Asp Ile Thr Asp Pro Gln Ser Val Gln Gln Ala Ala Lys Trp Val
                85                  90                  95

Glu Met His Val Lys Glu Ala Gly Leu Phe Gly Leu Val Asn Asn Ala
            100                 105                 110

Gly Val Ala Gly Ile Ile Gly Pro Thr Pro Trp Leu Thr Arg Asp Asp
        115                 120                 125

Phe Gln Arg Val Leu Asn Val Asn Thr Met Gly Pro Ile Gly Val Thr
    130                 135                 140

Leu Ala Leu Leu Pro Leu Leu Gln Gln Ala Arg Gly Arg Val Ile Asn
145                 150                 155                 160

Ile Thr Ser Val Leu Gly Arg Leu Ala Ala Asn Gly Gly Gly Tyr Cys
                165                 170                 175

Val Ser Lys Phe Gly Leu Glu Ala Phe Ser Asp Ser Leu Arg Arg Asp
            180                 185                 190

Val Ala His Phe Gly Ile Arg Val Ser Ile Val Glu Pro Gly Phe Phe
        195                 200                 205

Arg Thr Pro Val Thr Asn Leu Glu Ser Leu Glu Lys Thr Leu Gln Ala
    210                 215                 220

Cys Trp Ala Arg Leu Pro Pro Ala Thr Gln Ala His Tyr Gly Gly Ala
225                 230                 235                 240

Phe Leu Thr Lys Tyr Leu Lys Met Gln Gln Arg Ile Met Asn Leu Ile
                245                 250                 255

Cys Asp Pro Asp Leu Thr Lys Val Ser Arg Cys Leu Glu His Ala Leu
            260                 265                 270
```

```
Thr Ala Arg His Pro Arg Thr Arg Tyr Ser Pro Gly Trp Asp Ala Lys
        275                 280                 285

Leu Leu Trp Leu Pro Ala Ser Tyr Leu Pro Ala Ser Leu Val Asp Ala
    290                 295                 300

Val Leu Thr Trp Val Leu Pro Lys Pro Ala Gln Ala Val Tyr
305                 310                 315
```

SEQ ID NO: 2

```
   1 ccaggttttc cctcccttcc cccactcagc tgcaggaact ccttttttggg gtttggatct
  61 ggtatttttc tattcagctc cgagcttggc tctcctgggg aatcctggga gtgaaaggaa
 121 ggagctgggt ttatttgcat gtactggtag tcatttgcat cacatccaaa aatggccaaa
 181 attataaccc ctgattcttg gctgaactgg gactgctgca atggaatatt attcccggaa
 241 accaccccca actagctgga gctaatctcc tccctcctcc aacccccccat tttggcccag
 301 gcctacataa accaaaaaaa gctggaccat aaggtgaaaa ccctacaggt ccaggctgcc
 361 caatttgcca agcaaacagg ccattggatc gaaatggtga aaaacttcaa ccaggcactc
 421 aaggtgggcc atactcccta cctcaccacc ccaatcctgg gcccccattg gctgcctcca
 481 gtcaggttac ctcaggttta ggttaaggag gaagtagggt ggtcccagaa accccatcta
 541 tagccccagt gtcagaaaag gttgagaaag aaagaaaagc agttggtggg tccaagttaa
 601 agccttttcc aggagatgaa taaaacttat tccccaatgg aagccatagt ctacccattc
 661 tgattcctgg gtcccaactc ctctcccccct ttccaggaaa ttggggatgt ggagaatggg
 721 cttggagcat tgagctggaa atgcgcacca ttgccaatgc aatggaatat gtttacaaag
 781 ggcagctgca gtttgccccct tcctagcccc tgttccctcc cccaacccta tccctcctac
 841 ctcacccgca gggggaagga gggaggctga caagccttga ataaaaaaca agcctccgtt
 901 tttttgtggt gtgtttcaga gaggtaatag ctccagtgtc gggggtggga gtggaaggtt
 961 caaaggtggt ttccctgagg gacaggtacc ttttggggag agggtggaaa tagcttcctt
1021 ttactatccc aaatttttttt tcctccatgg cccttgtgca ggtgtttgtt aggcaagcag
1081 agggtgggag ttcccatccc tcctgagaga aggtcctagt agccctgccc caagcttcct
1141 aattcaggaa ttgtttccta cagaagagaa acaaggcaag tacacctggt ccccagctct
1201 ggctttctgc ctctccacgt gctcatggcc tctccccagg ctaactctaa gcagtgtcat
1261 gagtctgagc caggtgggag attaattcct gggggcactt cagggctgag aaggggggagg
1321 aatgacaggt ccagtaaccg ttaccaacag agcagtgcag ctgccatcct tgacagctcc
1381 ctcctccttg gagaccatga catagatggt caggaaccca ggctgagaaa gacagccaag
1441 gggtgggggg agcctaggca aatctggcct ctgccaagtc ctggcttcag ccaggcaagc
1501 tccagcctcc ctggctcctc ctcctcctca gtcctatccc caccctgtca cacatacact
1561 taatacgcct ggcatccaag tccacccact ccggactttg gccttagcag tagttagtgt
1621 gggaggctgg gaagactggg agcagtctct taaacaaaag caaaagaata agcttcgggc
1681 gctgtagtac ctgccagctt tcgccacagg aggtaagtgg atctgggagc tgggggaact
1741 gagaagacta gccagatatt acatgtattg ccaactcaaa actttcagct tttaacatgc
1801 ttcctcacac attatccccct ttgatcctcc acaactctga ggtggacctg gtgggtctta
1861 gccccacttg gtagatgaga aaataggttg agagagacag tgagatgctc agtatcacac
1921 agcaaacctc ttggccctat acatcattcc aaacacaaga cccaggttgc atatagaagg
1981 ttcagtgtcc ctggtttaga aggagaggtg gtgtgaggca agcaagaaga tgcctctgct
2041 gcactccagc ctgggcgaca gagtgagact ccatctcaaa aaaaaaaaaa aacgatgcct
2101 ctgctccata cagcaggtct gtacacagga tctggctcat gtggttttag ttaagttagc
2161 cacaaataca gggtctgccc acatctttgc tttgaacaga tgagccatgg ttggccaatt
2221 atctgccaac cagataattt ctcaatatgc tcacaccaga tgcttccagc tagggagggt
2281 attaggggaa agggcttgag ggccacagta aactggacaa gtttttctgc ccagcctagg
2341 ctgccacctg taggtcactt gggctccagc tatgtggctg cctcttctgc tgggtgcctt
2401 actctgggca gtgctgtggt tgctcaggga ccggcagagc ctgcccgcca gcaatgcctt
2461 tgtcttcatc accggctgtg actcaggctt tgggcgcctt ctggcactgc agctggacca
2521 gagaggcttc cgagtcctgg ccagctgcct gacccctcc ggggccgagg acctgcagcg
2581 ggtggcctcc tcccgcctcc acaccaccct gttggatatc actgatcccc agagcgtcca
2641 gcaggcagcc aagtgggtgg agatgcacgt taaggaagca ggtaagtatg gtagaccacc
2701 aggaatatgg tgtggggtgt cctgatcccc acagtcaccc caggagtcac ctgcaagggc
```

2761 tgtggtaagc taaagggaca atttgaggag aagcagtttt cagatgctcc caggaagaag
2821 agggagctgt gggagtgcct cacctacccc cagcatcctt ttcatctccc cacagggctt
2881 tttggtctgg tgaataatgc tggtgtggct ggtatcatcg gacccacacc atggctgacc
2941 cgggacgatt tccagcgggt gctgaatgtg aacacaatgg gtcccatcgg ggtcaccctt
3001 gccctgctgc ctctgctgca gcaagcccgg ggccgggtga tcaacatcac cagcgtcctg
3061 ggtcgcctgg cagccaatgg tgggggctac tgtgtctcca aatttggcct ggaggccttc
3121 tctgacagcc tgaggtgagg ggtacagggc tctgggttcc aggactaaca gcagcccact
3181 caacaaacgt gggccagcag aggtggttaa aatacagcac attggaatag ttaaaaagag
3241 acagtttagg gctaaacttc atgggttcaa tgaagtctac ccttatgtaa gctttgtgac
3301 cataagtaga ttacttctct ttacccattt ttaacgtgtt tgtttttttgt tttttgagat
3361 ggagtcttgc tctgtcgcca ggctggagtg cagtggcgcg atcttggctc accacaattt
3421 ccacccccgg ggttcaagcg attctcctgc ctcagcctcc cgagtagctg ggactacagg
3481 catgcgccac catgcctggc taatttttgt atttttagta gagacagggt ttcactatgt
3541 tggccaggtt ggtctcaaac tcctgacctc gtgatccgcc cacctcagcc tcccaaagtg
3601 ctgggattac aggtgtgagc caccacgccc ggccttgcct ctcgtctttta aacaataagg
3661 ttcaaagttc cgtgggagca caaaggagac atgatgagga caacgggagt tagggcctga
3721 gttttttttgg ttttttttttt ttaagcgttt tgctcttgtt gcctaggctg gagtgcaatg
3781 gcgagatctc agctcacagc aacccctgcc tctcaggttc atgtgattct cctgcctcag
3841 cctcccgatt agctgggctt acaggcacgt gccaccactc ccagctaaat ttttaggtag
3901 agatggagtt tataccatgt ggccagggtg ggtttgaatt cctgacctca cctgatccac
3961 cggaccggcc ttcccaaagt gctgggatta caggcatgag ccaccacaca cggcccaagg
4021 cctgagttct tagcaggagt ataaggcgcc taagcttagt ctaccttcta aggaagcctg
4081 cgtttgtcac catcactcag caaataaccg gaattgtctc ctgtctctca gccttaattt
4141 ttcaggcagc atcatgggac acatactttt agttttgaga caaggccttg ctctcaccca
4201 gggtggagtg cagtggtgca gtcacggccc actgaacttc aaactcctag gctcaagcag
4261 ctcaagcgat atccgcctca gcctcctgag tagctgagac cacaggcgcg tgccagcatg
4321 cctggctagt attttttttac agatggggtc ttgctgtggt gaccagactt gtctccaact
4381 cccggcctca agcgatgctt ccgcctgggc ctcccaaagt gttgggatta taggtgtgag
4441 ccactgcata ctggaacaca tactttatac ttgaattttt ttttatcccc ttccttcgtg
4501 ctcctaacct atacttggat ttctacatct gtgccagggc agtgggatgt atccccactt
4561 tccccatcag cttaccctcc agcaaatacg agactatacc cttcaatatc cagcactcag
4621 ggctcaacca tgtgttttgg gagcaaggga atggggttcc tctaggtcag gaatcggcaa
4681 actcagtact caagccagat ttggccagct gcctacaagc tgataatggt ttttttttatt
4741 tttaaatggt tacattgtaa actgttatat aagtacctga taatatcatt aattttgttt
4801 cttggcctgc catgcttaaa atattaactc tctggccctt taagaaaaaa acgtgctgac
4861 ccctgctcta gatcaaagaa aacaaacctc aaaaatactt tcctcccctct accccacttg
4921 acccttgtcc cggggcagta ggcatctccg tcaaaactct tgtccctggt ctgtggtaac
4981 tttctcagct ccccaaccca tgtccctcaa agtcccctcc ctatagggca agaacccagc
5041 aacttcgctc tgccccgact ctaggcggga tgtagctcat tttgggatac gagtctccat
5101 cgtggagcct ggcttcttcc gaacccctgt gaccaacctg gagagtctgg agaaaaccct
5161 gcaggcctgc tgggcacggc tgcctcctgc cacacaggcc cactatgggg gggccttcct
5221 caccaagtgt gagtagccag gcccacacag gggcacatga agggaaacaa gtaccagaaa
5281 ggccagtcct gcataagcct gctaggaggt gggtggggca cccagggcag ggttgagggt
5341 gaacaggatg ttacaanagt gcccaggcca tgtggaacct gcccactccc cacactgagg
5401 aggggactga gggtgacaag cccagggccc caaaaaacag tacctaanat gggctggagt
5461 gaggaaggga aactgattgc aaccacctat ggggctgcag acctgaaaat gcaacagcgc
5521 atcatgaacc tgatctgtga cccggaccta accaaggtga gccgatgcct ggagcatgcc
5581 ctgactgctc gacacccccg aacccgctac agcccaggtt gggatgccaa gctgctctgg
5641 ctgcctgcct cctacctgcc agccagcctg gtggatgctg tgctcacctg ggtccttccc
5701 aagcctgccc aagcagtcta ctgaatccag ccttccagca agagattgtt tttcaaggac
5761 aaggactttg atttatttct gcccccaccc tggtactgcc tggtgcctgc cacaaaataa
5821 gcactaacaa aagtgtattg tttaaaaaat aaaaagaagg tgggcagaaa tgtgcccagt
5881 ggaaggctga ccccatttaa gtgccaacta ctccaaaccg acatgctcac ggtctctggc
5941 ctgttcagtc cctgcaaaac agctagcacc cacagtgggg cgccagggaa ctgcctcaca
6001 tctacagctg cacgtcgggg agtggccatc aaagggcact ttaatacatt tcccttattt
6061 tctgaagggg agtaaggttg caattcagtg tctgtactgg gaatggtctt catatttctt
6121 gggggagaag agcaggtgat gagggttctg ggccaggctg ggtggcttcc atggaagaaa
6181 aggcaatatt cacataaatt ctcctgctaa ggacactgac cacacaggtg tgcaaggcaa
6241 cttatcatac ttcgaaagga gctggatccc ttgaggattg gccaggaagg gaggtgctgg
6301 gcccttagcg gtgcacagaa ggccaggaag

SEQ ID NO: 5

TAAGCTTCGG GCGCTGTAGT ACCTGCCAGC TTTCGCCACA GGAGGCTGCC ACCTGTAGGT
60

CACTTGGGCT CCAGCTATGT GGCTGCCTCT TCTGCTGGGT GCCTTACTCT GGGCAGTGCT
120

GTGGTTGCTC AGGGACCGGC AGAGCCTGCC CGCCAGCAAT GCCTTTGTCT TCATCACCGG
180

CTGTGACTCA GGCTTTGGGC GCCTTCTGGC ACTGCAGCTG GACCAGAGAG GCTTCCGAGT
240

CCTGGCCAGC TGCCTGACCC CCTCCGGGGC CGAGGACCTG CAGCGGGTGG CCTCCTCCCG
300

CCTCCACACC ACCCTGTTGG ATATCACTGA TCCCCAGAGC GTCCAGCAGG CAGCCAAGTG
360

GGTGGAGATG CACGTTAAGG AAGCAGGGCT TTTTGGTCTG GTGAATAATG CTGGTGTGGC
420

TGGTATCATC GGACCCACAC CATGGCTGAC CCGGGACGAT TTCCAGCGGG TGCTGAATGT
480

GAACACAATG GGTCCCATCG GGGTCACCCT TGCCCTGCTG CCTCTGCTGC AGCAAGCCCG
540

GGGCCGGGTG ATCAACATCA CCAGCGTCCT GGGTCGCCTG GCAGCCAATG GTGGGGGCTA
600

CTGTGTCTCC AAATTTGGCC TGGAGGCCTT CTCTGACAGC CTGAGGCGGG ATGTAGCTCA
660

TTTTGGGATA CGAGTCTCCA TCGTGGAGCC TGGCTTCTTC CGAACCCCTG TGACCAACCT
720

GGAGAGTCTG GAGAAAACCC TGCAGGCCTG CTGGGCACGG CTGCCTCCTG CCACACAGGC
780

CCACTATGGG GGGGCCTTCC TCACCAAGTA CCTGAAAATG CAACAGCGCA TCATGAACCT
840

GATCTGTGAC CCGGACCTAA CCAAGGTGAG CCGATGCCTG GAGCATGCCC TGACTGCTCG
920

ACACCCCCGA ACCCGCTACA GCCCAGGTTG GGATGCCAAG CTGCTCTGGC TGCCTGCCTC
980

CTACCTGCCA GCCAGCCTGG TGGATGCTGT GCTCACCTGG GTCCTTCCCA AGCCTGCCCA
1020

AGCAGTCTAC TGAATCCAGC CTTCCAGCAA GAGATTGTTT TTCAAGGACA AGGACTTTGA
1080

TTTATTTCTG CCCCCACCCT GGTACTGCCT GGTGCCTGCC ACAAAATA
1128